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Lipids and Lipoproteins

Evaluation of an immunoseparation method for quantitative measurement of remnant-like particle-cholesterol in serum and plasma

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▸ Abstract

Substantial evidence indicates that triglyceride-rich lipoprotein remnants are atherogenic. Additional research has, however, been limited by available methods for separation and quantification of remnants. We have evaluated an immunoseparation assay developed to measure cholesterol in remnant-like particles (RLP-C). This method uses monoclonal antibodies to human apolipoproteins B-100 and A-I to remove most of the apolipoprotein B-100-containing lipoproteins (namely LDL and nascent VLDL) and apolipoprotein A-I-containing lipoproteins (namely chylomicrons and HDL), leaving behind a fraction of triglyceride-rich lipoproteins, including chylomicron and VLDL remnants, both of which are enriched in apolipoprotein E. Cholesterol in the unbound fraction is measured with a sensitive enzymatic assay. The RLP-C concentration was highly correlated with total triglyceride-rich lipoproteins (sum of VLDL-cholesterol and IDL-cholesterol) separated by

ultracentrifugation and by polyacrylamide gel electrophoresis (*r* = 0.86 and 0.76, respectively). The within-run and run-to-run imprecision (CV) of the assay was ~6% and 10%, respectively. The assay was not affected by hemoglobin up to 5000 mg/L (500 mg/dL), bilirubin up to 342 mmol/L (20 mg/dL), glucose up to 67 mmol/L (1200 mg/dL), or ascorbic acid up to 170 mmol/L (3.0 mg/dL). In 726 subjects (men, n = 364; women, n = 362) in the US, the 75th percentiles of RLP-C concentration were 0.17 mmol/L (6.6 mg/dL) and 0.23 mmol/L (8.8 mg/dL) in sera obtained after overnight fasting or randomly, respectively. A group of 151 patients from nine US centers and one Canadian center with coronary artery atherosclerosis established by angiography had higher median RLP-C concentrations than 302 gender- and age-matched controls (*P* <0.05). We conclude that the RLP-C assay compares favorably to ultracentrifugation and electrophoresis and provides a convenient and economical approach to measure triglyceride-rich lipoprotein remnants in routine clinical laboratories.

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The importance of plasma triglycerides (TGs)[1] as a risk factor for atherosclerotic disease has been controversial. Whereas some epidemiological studies have suggested that serum TG concentration is an independent risk factor for coronary artery disease (CAD) (1)(2)(3), it has often been found to predict CAD in univariate models but not in multivariate models after adjustment for HDL-cholesterol (HDL-C) and other risk factors (4)(5)(6). In a recent Framingham study update, TGs were an independent risk factor for women but not for men (7). Metaanalysis of >50 000 subjects in 17 population-based prospective studies indicated that TGs are an independent risk factor for both men and women in the general population (8). One limitation of using plasma TG concentration as a predictor for CAD is that it does not separate nonatherogenic TG-rich lipoproteins, such as chylomicrons and nascent VLDL, from their potentially more atherogenic components, such as chylomicron and VLDL remnants. Studies in which lipoprotein remnants have been estimated have consistently demonstrated that increased plasma remnant concentrations are associated with the presence and progression of CAD (9)(10)(11)(12)(13).

General application of these research findings has been limited by lack of facile methods for quantification of VLDL and chylomicron remnants. Although lipoprotein remnants are adequately defined in functional terms, no straightforward methods have been developed to separate them from other TG-rich lipoproteins (14). Preparative and analytical ultracentrifugation (UC) have been used to separate VLDL and intermediate-density lipoprotein (IDL), designating the small VLDL and IDL as lipoprotein remnants. Smaller TG-rich lipoproteins have also been separated by nondenaturing polyacrylamide gel electrophoresis (PAGE) (15) or by electrophoresis in agarose gels, in which they have reduced mobility (16). These methods have limited applicability for routine analysis in clinical laboratories because they are labor-intensive, complex, and expensive.

Recently, a novel immunoseparation method for remnant lipoproteins was developed by Nakajima et al. (17). This method uses a monoclonal antibody to human apolipoprotein (apo) B-100 that recognizes an epitope near B-51 (18) to remove almost all LDL and most VLDL particles containing apo B-100

together with a monoclonal antibody to apo A-I to remove almost all HDL particles. Remaining unbound to these antibodies are principally all chylomicron remnants and a fraction of VLDL particles, both enriched in apo E, a characteristic of remnant lipoproteins (19). This unbound fraction is designated "remnant-like particles" (RLPs). The monoclonal antibodies are conjugated to Sepharose 4B to facilitate the separation of bound lipoproteins such as LDL and HDL from the RLP fraction. Cholesterol in the unbound fraction (RLP-C) is measured by an enzymatic cholesterol assay. With this immunoseparation method, researchers have found that RLP-C is increased in patients with CAD (20)(21), type III hyperlipoproteinemia (22), and diabetic nephropathy (23) and in patients undergoing hemodialysis (24)(25).

The objectives of this study were to evaluate the technical performance of this RLP-C assay and to assess the relationship between RLP-C and other atherogenic lipoproteins. A reference range of serum RLP-C for the US population was established, and the serum RLP-C concentrations were evaluated in patients with CAD confirmed by coronary angiography.

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study subjects

To study the effects of specimen type (serum or EDTA- or heparin-treated plasma), fasting status, and sample storage conditions on RLP-C measurement, RLP-C concentrations were measured in samples collected from 43 subjects with different lipid concentrations. The subjects participated in two visits: one after 12 h of fasting and the other within 4 h of their last meal. Two tubes of each specimen type were collected in predetermined order (serum, EDTA, heparin, EDTA, heparin, serum) from each subject during each phlebotomy session. The two tubes of same specimen type were combined for the RLP-C analysis.

To establish US reference ranges of serum RLP-C concentrations, 364 men and 362 women older than 17 years were recruited in four different parts of the country (Austin, TX; Brighton, MA; Miami, FL; and Phoenix, AZ). These subjects were free of symptoms and signs of CAD and of endocrine or metabolic disorders that may affect lipid metabolism. However, lipid and lipoprotein concentrations per se were not part of the inclusion or exclusion criteria. No subject took any medication that may alter lipid or lipoprotein metabolism. Each subject was asked to participate in two visits: one after 12 h of fasting and one at random in relation to the last meal (either fasting or nonfasting). Serum concentrations of RLP-C, total cholesterol (TC), TGs, and HDL-C were measured at each visit.

To evaluate the clinical utility of RLP-C in predicting CAD, 203 adult patients with >20% stenosis of at least one coronary artery at coronary angiography were recruited from nine medical centers in the US (Boston, MA; Jacksonville, FL; Lombard, IL; Santa Rosa, CA; Houston, TX; Minneapolis MN; Winter Park, FL; Ocala, FL; and Clearwater, FL) and one medical center in Canada (Montreal, PQ). Control subjects (n = 477) with similar ages to the CAD patients were recruited from the same four US centers that participated in the reference range study. Subjects with serum TG blank values >500 mg/L (50 mg/dL; because of heparin used during angiography before specimen collection), who did not complete

both visits, or who did not have a fasting specimen collected were excluded from the data analysis. The remaining population included 172 CAD patients and 469 controls.

All clinical protocols were approved by the Institutional Review Board. Informed consent forms were signed by all participating subjects.

materials

The RLP-C Immunoseparation Reagent kits were provided by Japan Immunoresearch Laboratories. The kit consists of immunoaffinity gel, cholesterol reagent, a cholesterol calibrator, and a Tris-HCl buffer solution (pH 7.4). The immunoaffinity gel consists of monoclonal antibodies to human apo B-100 (JI-H) and apo A-I (H-12) conjugated to Sepharose 4B beads as the solid phase (17). The cholesterol reagent contains ascorbate oxidase, cholesterol esterase, and cholesterol oxidase in R1, and horseradish peroxidase, 4-amino-antipyrine, and *N*-ethyl-*N*-(3-methylphenyl)-*N'*-succinyl-ethylenediamine in R2.

To monitor the imprecision of the RLP-C assay, fresh-frozen serum quality-control pools (RLP QC) were prepared and stored at -70 °C. They were supplemented with potassium EDTA (8 g/L) and sucrose (100 g/L) to preserve lipoprotein integrity during freeze-thaw cycles. Addition of sucrose has been shown to protect lipoproteins from damage during lyophilization (26). Diluted human serum pools were also prepared to monitor the imprecision of the cholesterol assay independent from the immunoseparation procedure (Low Cholesterol QC). Serum samples were diluted to cholesterol concentrations between 0.13 and 0.65 mmol/L (between 5 and 25 mg/dL), using normal saline with 50 g/L bovine serum albumin. EDTA and sucrose were also added into the diluted serum pools. The EDTA/sucrose-supplemented sera were then further diluted 61-fold with RLP buffer before freezing at -70 °C to simulate the dilution of samples during the RLP immunoseparation step. All frozen-serum QC pools were thawed and mixed on a shaker for 30 min at room temperature before use.

Hemoglobin and bilirubin (conjugated and unconjugated) were obtained from Kokusai Shiyaku Co., Ltd. (Interference Check-A Plus, cat. no. 5186). Glucose and ascorbic acid were obtained from Sigma Chemical Co.

rlp-c analysis

A 3-mm diameter stainless steel bead (Otsuka Electronics) was added to each microsample cup (part no. 140-668-0001, Boehringer Mannheim); 300 μL of the immunoaffinity gel and 5 μL of specimen were then added to each cup. The mixture was incubated at room temperature on a dedicated mixer with build-in magnetic bars effecting mixing by driving the beads up and down in each cup (18). After 2 h, the supernatant (unbound fraction) was transferred for cholesterol analysis in a Cobas MIRA S chemistry analyzer (Roche Analytical Instruments, Inc.). The cholesterol values were multiplied 61-fold to reflect dilution of the specimen at the immunoseparation step (Table 1).

View this table: [in this window] [in a new window]	**Table 1.** RLP-C test setup on the Cobas MIRA Analyzer.

technical evaluation of the rlp-c assay

The detection limit of the RLP-C assay was defined as 3 SD above the mean from 10 replicate analyses of the RLP buffer over 2 days. To evaluate linearity, a serum with an increased RLP-C concentration was mixed with a second serum with low RLP-C concentration or with normal saline in various proportions and then analyzed in duplicate. Within-run imprecision (CV) was evaluated by running the two RLP QC pools in 20 replicates. The run-to-run imprecision of the RLP-C assay was evaluated over a 3-month period on the same RLP QC pools by multiple analysts. The CV of the cholesterol assay was evaluated using the two Low Cholesterol QC pools without the immunoseparation step.

To compare the difference between serum and plasma in RLP-C measurement, specimens from 43 subjects were analyzed. Blood samples (fasting or nonfasting) were drawn into various Vacutainer Tubes (Becton Dickinson) in the following order: SST (for serum), EDTA, lithium heparin, EDTA, lithium heparin, SST. After the samples stood at room temperature for 30 min, serum or plasma were prepared by centrifugation to remove blood cells. The same specimen types were pooled and then aliquoted into cryogenic vials for storage at 4 °C or at -70 °C. TC, TGs (corrected for a glycerol blank), and HDL-C were measured in the fresh fasting sera. RLP-C was measured in all specimens within 4 h of collection and at specific intervals after storage.

Interference of hemoglobin, bilirubin, glucose, and ascorbic acid with the RLP-C analysis was evaluated by adding various concentrations of the respective interference substances or saline as control to two serum samples.

other lipid and lipoprotein analyses

TC and TGs were measured by CDC-standardized enzymatic methods. HDL-C was measured after precipitation of non-HDL lipoproteins by dextran sulfate (M_r 50 000)-Ca^{2+}. VLDL, IDL, and LDL were separated by UC at 250 000 *g* rpm using a Beckman Ti 42.2 rotor for 4 h or a Beckman 40.3 rotor for 18 h (27). Serum was centrifuged, and then the centrifuge tube was sliced with a Beckman tube slicer to yield a d <1.006 kg/L fraction (containing chylomicrons and VLDL) and a d ≥1.006 kg/L fraction (containing IDL, LDL, and HDL). The serum was also adjusted to a density of 1.019 kg/L with potassium bromide solution and then centrifuged to yield a d <1.019 kg/L fraction (containing chylomicrons, VLDL, and IDL) and a d ≥1.019 kg/L fraction (containing LDL and HDL). Cholesterol concentrations in each of the four fractions were measured by an enzymatic cholesterol assay. VLDL-cholesterol (VLDL-C) was calculated as [TC - cholesterol in d ≥1.006 kg/L lipoproteins]. IDL-C was calculated as [cholesterol in d ≥1.006 kg/L lipoproteins - cholesterol in d ≥1.019 kg/L lipoproteins]. LDL-C was calculated as [cholesterol in d ≥1.006 kg/L lipoproteins - HDL-C] or measured by the Direct LDL-Cholesterol method (Genzyme Corp.).

Lipoproteins were also separated and measured by PAGE (LipoPhor™ by Quantimetrix) (28). Lipoprotein(a) was measured by an immunoturbidimetric assay from INCSTAR in a Cobas FARA automated chemistry analyzer (Roche Analytical Instruments, Inc.).

statistical analyses

Imprecision of the assays was expressed as CV. Comparison between RLP-C and other lipoproteins was evaluated using the Pearson correlation. The difference among different specimen types was estimated

using ANOVA. The Mann–Whitney *U*-test was used to estimate the statistical difference between CAD patients and age- and gender-matched controls in their lipid and lipoprotein values because some of the values did not fit a gaussian distribution. A P value <0.05 was considered statistically different.

Results

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specimen type and stability

Fasting and nonfasting RLP-C concentrations in 43 simultaneously collected samples of serum, EDTA plasma, and heparin plasma were compared. Fasting serum TC, TG (corrected for glycerol blank), and HDL-C concentrations were 5.91 ± 1.50 mmol/L (228 ± 58 mg/dL), 2.80 ± 4.33 mmol/L (248 ± 383 mg/dL), and 1.27 ± 0.44 mmol/L (49 ± 17 mg/dL), respectively. To allow the use of ANOVA, a parametric statistical analysis method that is based on the assumption of gaussian distribution of data, five samples with TG concentrations >4.52 mmol/L (400 mg/dL) were analyzed separately. Mean fasting RLP-C concentrations in serum, EDTA plasma, and heparin plasma were 0.17 ± 0.09 mmol/L (6.7 ± 3.4 mg/dL), 0.19 ± 0.08 mmol/L (7.4 ± 2.9 mg/dL), and 0.18 ± 0.08 mmol/L (6.8 ± 3.1 mg/dL), respectively. Mean nonfasting RLP-C concentrations were 0.21 ± 0.11 mmol/L (8.2 ± 4.1 mg/dL), 0.23 ± 0.10 mmol/L (8.7 ± 3.9 mg/dL), and 0.21 ± 0.10 mmol/L (8.3 ± 3.9 mg/dL), respectively, for the three types of specimens. Mean RLP-C concentrations in fasting or nonfasting EDTA plasma samples were slightly higher than in serum or heparinized plasma (P <0.05).

We evaluated the effects of two different storage conditions (4 °C and -70 °C) on RLP-C values. RLP-C concentrations in the nonfasting samples tended to increase when stored at 4 °C (Fig. 1). The increase of mean fasting RLP-C concentrations was <0.03 mmol/L (1.0 mg/dL) within 1 week of sample collection. Freezing at -70 °C prevented the gradual increase seen in both fasting and nonfasting samples with TG concentrations <4.52 mmol/L (400 mg/dL) for up to 3 months (Fig. 1). Fasting RLP-C concentrations rose after freezing in three of the five samples with TG concentrations >4.52 mmol/L (400 mg/dL).

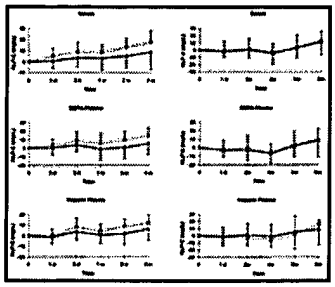

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Figure 1. Change of mean fasting (♦) and nonfasting (□) RLP-C concentrations in serum, EDTA plasma, and heparin plasma from 38 subjects after storage at 4 °C up to 4 weeks *(left)* and at -70 °C up to 6 months *(right)*.

technical performance of the rlp-c assay

The detection limit, defined as 3 SD above the mean value for the RLP buffer, was 0.08 mmol/L (3.1 mg/dL). The assay was linear up to 3.37 mmol/L (130 mg/dL) when serum was used as the diluent or to 2.33 mmol/L (90 mg/dL) when normal saline was used as the diluent.

The within-run CV of the RLP-C assay was 6.1% at 0.13 mmol/L (4.9 mg/dL) and 3.5% at 0.51 mmol/L (19.8 mg/dL). The within-run CV (n = 20) of the cholesterol assay, independent of immunoseparation, was 4.7% at 0.22 mmol/L (8.6 mg/dL) and 1.6% at 0.67 mmol/L (26.0 mg/dL; Table 2). The run-to-run CV over a representative 3-month period (n = 36) was 10.2% at 0.15 mmol/L (5.9 mg/dL) and 5.6% at 0.51 mmol/L (19.7 mg/dL). The run-to-run CV attributable to the cholesterol assay alone during the same period was 5.7% at 0.23 mmol/L (8.7 mg/dL) and 3.3% at 0.62 mmol/L (23.9 mg/dL; Table 2). The representative run-to-run CV was ~10% at 85 mg/L (8.5 mg/dL) for VLDL-C plus IDL-C by UC (n = 79) and 13% at 90 mg/L (9.0 mg/dL) for VLDL-C by LipoPhor (n = 64).

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Table 2. Within-run and run-to-run imprecision of RLP-C assay and cholesterol assay alone.

Hemoglobin up to 5000 mg/L (500 mg/dL), bilirubin (both conjugated and unconjugated) up to 342 mmol/L (20 mg/dL), glucose up to 66.6 mmol/L (1200 mg/dL), and ascorbic acid up to 170 mmol/L (3 mg/dL) did not interfere with the RLP-C assay. We showed previously that the binding capacity for HDL, expressed as its cholesterol concentration, was 2.59 mmol/L (100 mg/dL) (18). Above this concentration, a substantial amount of HDL appeared in the RLP fraction. By contrast, there was no clear cut point at which a relevant amount of LDL escaped antibody binding. Instead, the monoclonal antibody JI-H gradually lost its binding capacity with increased LDL (measured as LDL-C). Specifically, ~3% and 5% of the LDL were present in the unbound fraction in samples with LDL-C concentrations of 5.18 mmol/L (200 mg/dL) and 7.77 mmol/L (300 mg/dL), respectively (18).

relationships between rlp-c and other lipids and lipoproteins

To evaluate the relationship between RLP-C and other lipoproteins, we compared RLP-C with other TG-rich lipoprotein fractions separated by either UC or by PAGE (e.g., LipoPhor) in 157 CAD patients and 314 age- and gender-matched controls. The correlation coefficients *(r)* between RLP-C and VLDL-C were 0.88 and 0.77 for the VLDL separated by UC and LipoPhor, respectively (Fig. 2). RLP-C also moderately correlated with serum IDL-C by UC ($r = 0.46$) or by LipoPhor ($r = 0.59$; Fig. 2). The correlation coefficients between RLP-C and total TG-rich lipoproteins (VLDL-C + IDL-C) were 0.86 and 0.76 (measured by UC and LipoPhor, respectively). RLP-C was also highly correlated with serum TG ($r = 0.87$) but poorly correlated with TC ($r = 0.36$) and LDL-C ($r = 0.18$). There was no correlation between RLP-C and lipoprotein(a) ($r = -0.06$).

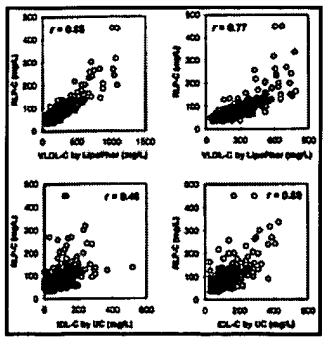

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Figure 2. Correlation between RLP-C and VLDL *(top panels)* and IDL *(bottom panels)* separated by UC *(left panels)* or PAGE (LipoPhor; *right panels*) in sera from 471 subjects.

Pearson correlation coefficients *(r)* are shown in each panel.

The lower correlation between RLP-C and IDL-C than with VLDL-C was supported by the investigation of the distribution of RLP-C in different lipoprotein fractions separated by UC. In 21 sera examined, the concentration of RLP-C in the IDL fraction, calculated as (RLP-C in the d <1.019 kg/L fraction - RLP-C in the d <1.006 kg/L fraction), was negligible except in samples with TG concentrations >5.65 mmol/L (500 mg/dL; Fig. 3). RLP-C in the d <1.006 kg/L fraction was highly correlated with serum TG concentrations (r = 0.95). At higher serum TG concentrations, a larger percentage of RLP-C was in the d <1.006 kg/L fraction.

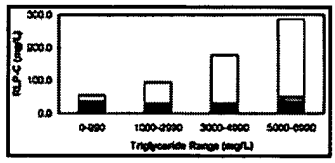

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Figure 3. Distribution of RLP-C in different hydrated densities (kg/L) in serum samples segregated by TG concentration.

Serum samples were centrifuged at densities of 1.006 kg/L and 1.019 kg/L, respectively, to obtain d <1.006 kg/L (□), 1.006 kg/L < d < 1.019 kg/L (▣), and d >1.019 kg/L (■) fractions. RLP-C was measured in each of the four fractions. RLP-C concentrations in the 1.006–1.019 kg/L fraction were calculated by subtracting the RLP-C in the d <1.006 kg/L fraction from the RLP-C in the d <1.019 kg/L fraction.

reference ranges of rlp-c in a us population

In 364 healthy men and 362 healthy women recruited from four US centers, median fasting TC, TG, LDL-C, and HDL-C concentrations were 5.21 mmol/L (201 mg/dL), 1.10 mmol/L (97 mg/dL), 3.34 mmol/L (129 mg/dL), and 1.22 mmol/L (47 mg/dL), respectively. The 25th through 75th percentiles were 4.48–5.96 mmol/L (173–230 mg/dL) for TC, 0.80–1.63 mmol/L (71–144 mg/dL) for TGs, 2.77–3.99 mmol/L (107–154 mg/dL) for LDL-C, and 1.01–1.45 mmol/L (39–56 mg/dL) for HDL-C. The median RLP-C concentrations were 0.13 mmol/L (5.1 mg/dL) in fasting samples and 0.16 mmol/L (6.2 mg/dL) in random samples. The 25th-75th-95th percentile concentrations were 0.10-0.17-0.32 mmol/L (3.9-6.6-12.3 mg/dL) in fasting samples and 0.13-0.23-0.46 mmol/L (4.9-8.8-17.6 mg/dL) in random samples.

There was a significant difference in RLP-C concentrations between men and women and between

different age groups (Table 3). In fasting samples, there was no difference in RLP-C concentrations among the three age groups tested for the men (18–34, 35–54, and ≥55 years). RLP-C concentrations increased with age in women. The median RLP-C concentration in women 55 and older was no different from that in men of the same age group. In contrast, random RLP-C concentrations increased with age in both men and women. In addition to the effects of gender and age, fasting and random RLP-C concentrations were higher in Hispanics (n = 173) and lower in African Americans (n = 67) than in Caucasians (n = 475). The median fasting RLP-C concentrations in the three populations were 0.14 mmol/L (5.4 mg/dL), 0.10 mmol/L (4.0 mg/dL), and 0.13 mmol/L (5.1 mg/dL), respectively.

View this table: [in this window] [in a new window]	**Table 3.** Comparison of RLP-C concentrations in different gender and age groups.

rlp-c in cad patients and controls

Of the 172 eligible CAD patients, 161 (94%) were Caucasians, 7 (4%) were African Americans, 2 (1%) were Hispanics, and 2 (1%) were other races. In comparison, Caucasians accounted for only 58% of the control population of the clinical utility study. To provide vigorous comparison between the CAD and control groups, we matched each Caucasian CAD patient with two Caucasian controls, either from the reference range study or the clinical utility study, by their gender and age (±1 year). The average age was 63.4 ± 8.6 years for both groups; 77% were men and 23% were women.

Median fasting and random RLP-C concentrations were significantly higher in CAD patients than in controls (P <0.05 and P <0.001, respectively; Table 4). Serum TG concentrations, fasting or random, were also significantly higher in CAD patients. HDL-C concentrations were significantly lower in CAD patients. LDL-C concentrations were slightly lower in CAD patients. There were no differences between the two groups in serum TC concentrations.

View this table: [in this window] [in a new window]	**Table 4.** RLP-C and other lipid/lipoprotein concentrations in patients with CAD and their age- and gender-matched controls.

Discussion

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We have evaluated the technical performance and clinical utility of the RLP-C assay. The assay in principle is the same as described previously by Nakajima et al. (17); however, the immunoseparation step and cholesterol assay have been modified to be easier to use in routine laboratories (18). Historically, lipoprotein remnants have been estimated mainly after separation of TG-rich lipoproteins by UC or PAGE. UC is considered the reference method for isolating lipoprotein classes.

This method, however, requires instrumentation not readily available in routine clinical laboratories and does not specifically separate lipoprotein remnants. Furthermore, the procedure is skill-dependent, labor-intensive, and has slow throughput. Although the instruments needed for PAGE are relatively inexpensive and available in laboratories performing electrophoresis, the ability to detect low concentrations of lipoprotein remnants or small changes in their concentrations is limited by the poor sensitivity of the method (28). Subjective interpretation of the electrophoretic pattern and identification of "IDL" bands (a broad "mid-band" between VLDL and LDL) may cause large variations in quantifying this putative remnant fraction, which may also contain lipoprotein(a). By contrast, the RLP-C assay requires only a small mixing device for the isolation of lipoprotein remnants (18), and the cholesterol concentration in the RLP fraction can be measured on any automated chemistry analyzer with an "open design" system and with a precise optical system. Analyzers with a "closed design" system (such as Johnson & Johnson Vitros series chemistry analyzers) are not suitable for RLP-C measurement because the cholesterol measurement with these analyzers lacks the sensitivity required. In comparison with the UC and PAGE methods, the RLP-C assay has a higher throughput and lower complexity. The run-to-run precision of the RLP-C assay is comparable with the UC or PAGE methods.

Lipoprotein remnants are a group of highly heterogeneous particles varying in size, hydrated density, electrophoretic mobility, chemical composition, and recognition by receptors (14). Each of the available separation methods measures lipoprotein remnants on the basis of different physical and chemical properties. UC separates lipoproteins by their hydrated density. Researchers typically have used IDL [hydrated density between 1.006 and 1.019 kg/L, or Svedberg flotation rates (Sv) of 12–20 units] as a surrogate to reflect remnant status because sequential hydrolysis of VLDL leads to a group of smaller, more dense, and cholesteryl ester-rich lipoprotein remnants. Chylomicron remnants, with a size and density generally larger than those of VLDL, do not appear in appreciable amounts as lipoproteins in the 1.006–1.019 kg/L density range. Some investigators have suggested that lipoproteins of Sv between 12 and 60 units reflect remnant status better than lipoproteins of Sv between 12 and 20 units (29). This range of lipoproteins includes both IDL ($1.006 < d < 1.019$ kg/L) and smaller VLDL particles found in the $d < 1.006$ kg/L fraction.

In contrast to UC, PAGE separates lipoproteins by their molecular sizes. With this method, lipoprotein remnants are often measured as "IDL", which includes any lipoprotein particles with the a size between VLDL and LDL (28). In the population studied, IDL measured by LipoPhor correlates only moderately with IDL isolated by UC, with a Pearson correlation coefficient of 0.57 (data not shown). In samples from the type III hyperlipoproteinemic patients, IDL-C measured by the two methods are highly correlated, with a Pearson correlation coefficient of 0.93 (data not shown).

The RLP-C assay separates lipoprotein remnants on the basis of their surface apo components. apo A-I-containing lipoproteins (namely HDL and chylomicrons) are removed by anti-apo A-I; most apo B-100-containing lipoproteins (namely LDL and most VLDL) are removed by anti-apo B-100 (JI-H) (17). This assay measures mainly chylomicron remnants and larger VLDL remnants (19). These particles are rich in apo E as compared with the bound VLDL (19)(30). However, in samples from patients with diabetic (18) and type III hyperlipoproteinemia (22), particles of IDL size are found in the RLP fraction. In our nondiabetic subjects who did not have type III hyperlipoproteinemia, the IDL fraction (1.006 kg/L

< d < 1.019 kg/L) contained little RLP-C (Fig. 3). RLP-C is thus better correlated with total TG-rich lipoproteins (VLDL + IDL) than with IDL-C alone (r = 0.86 vs 0.46 by UC or r = 0.76 vs 0.59 by LipoPhor (Fig. 2).

Although IDL concentrations have been shown to predict the presence (10) and progression (31) of CAD in some studies, evidence that these particles are more atherogenic than other TG-rich lipoproteins is limited. Lipoprotein remnants with d <1.006 kg/L, on the other hand, have been found to possess proatherogenic characteristics: they increase permeability of the endothelial barrier (32) and are taken up by macrophages by receptor-mediated mechanisms (33)(34). Furthermore, lipoproteins isolated from human atherosclerotic plaque structurally resemble TG-rich lipoprotein remnants (35)(36). Clinical studies have also suggested that the concentration of remnants in the VLDL plus IDL range predict coronary atherosclerosis lesion progression (11)(13) or clinical coronary heart disease events (11) better than LDL.

RLPs isolated by the immunoaffinity mixed gel have been demonstrated to have biological properties of atherogenic lipoprotein remnants, including inhibition of endothelium-dependent vasorelaxation (37)(38)(39), enhancement of platelet aggregation in whole blood (40)(41), and uptake by macrophages without modification (42)(43). All of these properties of RLPs are potentially proatherogenic. In addition to in vitro evidence, clinical studies have also demonstrated that RLP-C concentrations are higher in CAD patients (17)(20)(21). Serum RLP-C concentrations have also been found to be substantially higher in patients with non-insulin-dependent diabetes mellitus and microalbuminuria (23), undergoing chronic hemodialysis (24), or with intermittent claudication (44).

To help us evaluate the clinical utility of the RLP-C assay in North American populations, we conducted a multicenter program to establish reference ranges and to compare RLP-C concentrations in patients with CAD and in controls with similar ages. Consistent with other studies (17)(20)(21), RLP-C concentrations, particularly in random samples, were higher in Caucasian CAD patients than in age- and gender-matched Caucasian controls (Table 4).

Because of the large size of chylomicron-derived particles in nonfasting samples, VLDL-C cannot be calculated with reasonable accuracy in the postprandial state. To permit estimation of LDL-C, plasma lipid analyses are ordinarily performed on fasting samples in the clinical laboratory. A potential disadvantage of this practice is that it may reduce the opportunity to detect defects in lipoprotein remnant removal. It has been hypothesized that atherosclerotic disease can result from postprandial accumulation of remnant particles (45)(46). Previous studies have shown that postprandial RLP-C concentrations are substantially increased above fasting concentrations in CAD patients but not in healthy controls, where the fasting concentrations were considered within reference values in both groups (47)(48). Random sampling provides an alternative to cumbersome fat-loading studies in measuring postprandial RLP-C concentrations.

In summary, we have evaluated a novel immunoseparation method for the quantitative estimation of a lipoprotein remnant fraction. The RLP-C immunoseparation method is faster and easier to use than laboratory methods based on UC or electrophoresis. Additional clinical studies are needed to establish the usefulness of this novel assay.

Footnotes

[1] Nonstandard abbreviations: TG, triglyceride; CAD, coronary artery disease; HDL-C, HDL-cholesterol; UC, ultracentrifugation; IDL, intermediate-density lipoprotein; PAGE, polyacrylamide gel electrophoresis; apo, apolipoprotein; RLP, remnant-like particle; RLP-C, remnant-like particle-cholesterol; TC, total cholesterol; QC, quality control; VLDL-C, VLDL-cholesterol; IDL-C, intermediate-density lipoprotein-cholesterol; and LDL-C, LDL-cholesterol.

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Comparison of laboratory parameters as risk factors for the presence and the extent of coronary or carotid atherosclerosis: the significance of apolipoprotein B to apolipoprotein all ratio.

Cerne D, Ledinski G, Kager G, Greilberger J, Wang X, Jurgens G.

Institute of Clinical Chemistry and Biochemistry, University Medical Centre of Ljubljana, Slovenia.

We compared several "new" risk factors (autoantibodies to oxidatively modified low density lipoprotein (LDL), sialic acid content of LDL, bilirubin and C-reactive protein) with "conventional" risk factors (apolipoprotein (apo) AI, AII and B, lipoprotein(a), triglycerides, and total, LDL and high density lipoprotein (HDL) cholesterol) for the presence and the extent of coronary or carotid atherosclerosis. Forty male patients with angiographically proven coronary atherosclerosis and 31 male patients with ultrasound-proven extracranial carotid atherosclerosis were compared to 40 age matched (53+/-5 years) healthy males as control subjects, with negative parental history of atherosclerosis, no clinical signs of systemic or organ-related ischemic disease and normal extracranial carotid arteries. The apo B/apo All ratio most powerfully indicated the presence and the extent of coronary or carotid atherosclerosis. Elevated lipoprotein(a) contributed significant additional information in the assessment of the atherosclerotic risk. Increase in C-reactive protein indicated the presence (but not the extent) of coronary or carotid atherosclerosis with a similar power as lipoprotein(a). Decreased values of total bilirubin indicated the presence of atherosclerosis only in smokers. Autoantibodies to oxidatively modified LDL additionally described the atherosclerotic process, but were less important than apolipoproteins, lipoprotein(a), C-reactive protein or bilirubin. Sialic acid content of LDL added no information to the parameters discussed above. We demonstrated that in male patients apolipoproteins, especially the apo B/apo All ratio, were better indicators of the presence and the extent of coronary or carotid atherosclerosis than C-reactive protein, bilirubin, autoantibodies to oxidatively modified LDL or sialic acid content of LDL.

PMID: 10987202 [PubMed - indexed for MEDLINE]

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Comparison of various lipid, lipoprotein, and bilirubin combinations as risk factors for predicting coronary artery disease[*1]

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Abstract

Studies were performed to determine if serum bilirubin, when combined with various lipid and lipoprotein risk factors, enhances our ability to predict coronary artery disease (CAD). This hypothesis was tested in a retrospective study of 644 middle-aged males who had undergone coronary angiography. The traditional risk factors of cholesterol, high density lipoprotein cholesterol (HDL-C), cholesterol/HDL-C ratios, triglycerides, age, cigarette smoking, and systolic blood pressure were tested by discriminant analysis, as were various cholesterol/bilirubin, cholesterol/(HDL-C+bilirubin), and low-density lipoprotein cholesterol (LDL-C)/(HDL-C+bilirubin) ratios. Each of these bilirubin-containing ratios was found to be an independent risk predictor when tested with the traditional risk factors. When the LDL-C/(HDL-C+bilirubin) ratio was included with the traditional risk predictors, it improved the prediction of severe CAD from 28.4 to 35.3% and the overall correct classification of CAD from 68.3 to 71.1%. When the 75th percentile was used as a cut-point, the diagnostic sensitivities obtained with cholesterol/(HDL-C+bilirubin) ratios (52.1%) and LDL-C/(HDL-C+bilirubin) ratios (51.7%) were better than those obtained with cholesterol/HDL-C ratios (40.4%) (P=0.033 and 0.048, respectively). LDL-C/(HDL-C+bilirubin) ratios also improved the prediction of severe CAD over those obtained with LDL-C/HDL-C ratios (43.4%); however, the changes were not statistically significant (P=0.096). If confirmed in

other populations, serum bilirubin may be combined with LDL-C/HDL-C ratios, cholesterol/HDL-C ratios, cholesterol, or with various apolipoproteins to improve the prediction of CAD.

Author Keywords: Bilirubin; Cholesterol; HDL-cholesterol; Coronary artery disease; Risk factors; Antioxidants; Prediction

*1 The views expressed in this article are those of the authors and do not reflect the official policy of the Department of Defense or other Departments of the US Government.

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Lipid Profile

Related tests: Cholesterol, HDL, LDL, Triglycerides

What is a lipid profile?

The lipid profile is a group of tests that are often ordered together to determine risk of coronary heart disease. The tests that make up a lipid profile are tests that have been shown to be good indicators of whether someone is likely to have a heart attack or stroke caused by blockage of blood vessels ("hardening of the arteries").

What tests are included in a lipid profile?

The lipid profile includes total cholesterol, HDL-cholesterol (often called good cholesterol), LDL-cholesterol (often called bad cholesterol), and triglycerides. Sometimes the report will include additional calculated values such as HDL/Cholesterol ratio or a risk score based on lipid profile results, age, sex, and other risk factors.

How is a lipid profile used?

The lipid profile is used to guide providers in deciding how a person at risk should be treated. The results of the lipid profile are considered along with other known risk factors of heart disease to develop a plan of treatment and follow-up.

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Arteriosclerosis, Thrombosis, and Vascular Biology

(*Arteriosclerosis, Thrombosis, and Vascular Biology.* 1996;16:250-255.)

Articles

Higher Serum Bilirubin Is Associated With Decreased Risk for Early Familial Coronary Artery Disease

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Abstract

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Abstract Mildly increased serum bilirubin has recently been suggested as a protective factor, possibly reducing the risk of coronary artery disease (CAD) by acting as an antioxidant. We tested this hypothesis by examining serum bilirubin concentrations and other coronary risk factors in 120 men and 41 women with early familial CAD and 155 control subjects. At screening, both cases and control subjects were 38 to 68 years old. Early familial CAD patients had experienced myocardial infarction, coronary artery bypass grafting, or coronary angioplasty by age 55 years for men and 65 for women and had another sibling similarly affected. The average total serum bilirubin concentration was 8.9±6.1 μmol/L in cases and 12.4±8.1 μmol/L in control subjects (P=.0001 for difference). In univariate analysis stratified by sex, serum bilirubin was strongly and inversely related to CAD risk, with relative odds of 0.4 to 0.1 (relative to the lowest quintile, P=.04 to .00001) in both men and women as bilirubin increased into the upper two quintiles. Multiple logistic regression analysis was performed including age, sex, smoking, body mass index, diabetes, hypertension, plasma measured LDL

cholesterol, HDL cholesterol, triglycerides, and serum bilirubin as potential risk factors. Bilirubin entered as an independent protective factor with an odds ratio of 0.25 (P=.0015) for an increase of 17 μmol/L (1 mg/dL). The standardized logistic regression coefficient for bilirubin was -.33 compared with -.34 for HDL, suggesting that the protective effect of bilirubin on CAD risk in the population is comparable to that of HDL cholesterol. A history of cigarette smoking was associated with significantly lower serum bilirubin concentration and appeared to attenuate the protective effect of bilirubin.

Key Words: serum bilirubin • coronary artery disease • risk factors • case-control studies • genetics

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Serum bilirubin acts as a natural antioxidant in several in vitro systems.[1] [2] [3] [4] [5] [6] Furthermore, bilirubin, especially with albumin, appears to be cytoprotective.[7] [8] [9] Recent in vitro evidence suggests that LDL is protected from oxidation by bilirubin,[10] possibly acting with vitamin E as a co-antioxidant.[11] Although there is growing epidemiological evidence that other antioxidants, particularly vitamin E[12] [13] and some flavonols,[14] [15] [16] [17] protect against coronary artery disease (CAD), similar evidence that serum bilirubin may be protective is much more limited. The only epidemiological study to examine CAD risks associated with bilirubin was conducted among male Air Force pilots referred for coronary angiography. Stenoses of 50% or more were significantly associated with lower serum bilirubin concentrations independent of other CAD risk factors.[18]

We have been examining potential coronary risk factors in men and women with early familial CAD. Such patients tend to have a high prevalence of recognized coronary risk factors. They may be a particularly suitable group for examining the effects of novel risk factors because the high risk associated with a positive family history of CAD is not adequately explained by standard coronary risk factors.[19] [20] [21] [22] [23] [24] Accordingly, we have examined the CAD risks associated with graded levels of serum bilirubin in a representative group of early familial CAD cases and control subjects. This is the first study to examine this issue in women as well as in men.

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Early familial CAD patients included 120 men and 41 women who had survived a myocardial infarction, percutaneous transluminal angioplasty, or coronary artery bypass grafting before age 55 years for men and 65 for women. Each of these cases had a sibling confirmed to have early CAD by the same definition. Only unrelated CAD cases were used in the present

study. These were selected as the sibling with the earliest onset of CAD in families with multiple screened siblings. More than 90% of patients with early familial CAD who were invited to our clinic were screened. Given this high recruitment rate, the sample is probably quite representative of families with multiple cases of early CAD.

Control subjects comprised 85 men and 70 women who were ascertained from a random population sampling or who were spouses of hypertensive siblings who had participated in previous studies in our clinic. Both groups may be considered representative of a general population of adults with children in high school as they were selected from family health questionnaires filled out by high school students with the help of their parents as part of an ongoing program supported in part by the Utah Department of Health. Control subjects from the two sources were confirmed to have comparable risk factor distributions before they were combined into one group. Further details of the ascertainment methods have been published.[25] Nearly all cases and control subjects were white, reflecting the diverse and representative northern European ancestry of most Utah residents, in whom inbreeding is minimal.[26] [27]

Ages for both cases and control subjects were restricted to 38 to 68 years, but control subjects were not individually matched for age or sex. This study was approved by the Institutional Review Board of the University of Utah Medical Center. All subjects signed informed consent before participating.

We defined early familial CAD as the presence of two or more first-degree relatives with onset of CAD by age 55 years in men and 65 in women. CAD included prior diagnosis of myocardial infarction, percutaneous transluminal angioplasty, or coronary artery bypass graft. We considered hypertension to be present if a patient was taking antihypertensive medication with a prior physician diagnosis of hypertension or if the mean of two supine diastolic pressures taken with an automated blood pressure machine (Dynamap, Critikon) was greater than 95 mm Hg. Diabetes was considered present if a prior physician diagnosis had been made or if the fasting serum glucose on screening was greater than 7.77 mmol/L (140 mg/dL). For most analyses cigarette smoking was dichotomized into "ever" or "never" (since many patients quit after their first episode of CAD), with ever smoking defined as having smoked daily for 1 year or more.

Blood samples were collected in the morning after 12 to 16 hours of fasting and were prepared according to guidelines of the Lipid Research Clinics Program *Manual of Laboratory Operations*.[28] Lipids were measured by a microscale procedure developed in our laboratory.[29] Our lipid laboratory is certified by the Centers for Disease Control and Prevention in Atlanta, Ga. Total serum bilirubin was analyzed with a diazotized sulfanilic acid reagent and a commercial colorimetric method on a FARA II autoanalyzer (Roche).[30]

Statistical Analyses

The SAS statistical software package was used for data analysis (SAS Institute, Inc). Statistical analyses on triglycerides (which were significantly skewed to the right) were done

after logarithmic transformation to normalize the distribution. After transformation, the distribution was normally distributed as shown by the Kolmogorov D statistic. Analyses with bilirubin were performed both with and without log transformation. Univariate comparisons between cases and control subjects of qualitative variables (such as cigarette smoking, hypertension, or diabetes) were performed with the x^2 test and Fisher's exact test. For univariate comparisons of continuous quantitative variables, we used Student's *t* test. To evaluate potential confounding or interrelations between bilirubin and other variables, we used Pearson's correlation and ANCOVA (with the SAS GLM procedure). Unconditional stepwise multiple logistic regression was used to estimate risk associated with changes in bilirubin after correction for other recognized risk factors.

We did not select individually matched control subjects for several reasons. Of primary importance in a case-control design is that control subjects be representative of the general population from which the cases were selected and that the cases be representative of all similar cases. Age range should be comparable (since prevalence of several risk factors is age dependent). Given these prerequisites, individual matching is not necessary to control for confounding. An analysis stratified by the confounding factor (sex, for example) will provide equally valid results whether applied to matched or unmatched data. In fact, counter to intuitive expectation, matching can introduce confounding where none previously existed if the exposure variable to be tested is associated with the matching variable.[31] To avoid this bias, matched data can be analyzed only after stratifying by the matching variable or variables. In multiple logistic regression, because the prevalence of the matched variable among control subjects becomes "conditioned" on the distribution in the cases, a "conditional" analysis must be applied rather than "unconditional" regression. Furthermore, variables used for matching cannot be evaluated for associated risks. For example, if cases and control subjects had been matched by sex, the relative risk associated with male sex could not be calculated. Nevertheless, only after we found a similar pattern of risk association in each sex analyzed separately did we combine them for a grouped analysis. Finally, while matching can lead to improved statistical efficiency (not greater validity) in data analysis (by ensuring that different strata have sufficient numbers for meaningful comparisons), matching can add considerable cost if new control subjects must be recruited; it is usually not necessary when adequate numbers of cases and control subjects are available.[31]

Results

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Clinical characteristics of cases and control subjects are given in Table 1. Although our patients with CAD were significantly older than control subjects at the time of screening, their mean age at clinical onset of CAD was 46.5±6.6 years, 2 years younger than the control subjects (P=.0036). There were fewer women among the cases compared with control subjects. In addition, hypertension and diabetes were much more prevalent among the cases. This remained true even when only diagnoses made by the age of onset of CAD among the cases

were included (42.2% prevalence of hypertension by age at CAD diagnosis, 13.0% for diabetes, both $P<.00001$ compared with control subjects). Cases had significantly greater body mass index (kilograms per meter squared); blood pressures; and levels of serum glucose, plasma total cholesterol, triglycerides, and VLDL cholesterol. Plasma HDL cholesterol was lower among cases. Surprisingly, measured plasma LDL cholesterol levels were not different in cases versus control subjects. However, more than half (52%) of the cases were being treated for high cholesterol (34% were taking drugs), whereas only 6% of the control subjects were on a diet and none were taking lipid-lowering medication. These results were essentially the same for women and men considered separately, as reported previously.[32]

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Table 1. Clinical Characteristics of Cases and Control Subjects

The distributions of serum bilirubin concentration in cases and control subjects are shown separately for men and women in Fig 1. Among cases, the bilirubin distributions appeared to be shifted to lower levels compared with control subjects. Furthermore, there were no cases with a serum bilirubin concentration higher than 31 μmol/L (1.8 mg/dL), whereas six control subjects had levels in this range. The highest level seen in control subjects was 54.5 μmol/L (3.2 mg/dL). None of these higher bilirubin levels were associated with abnormal liver function tests.

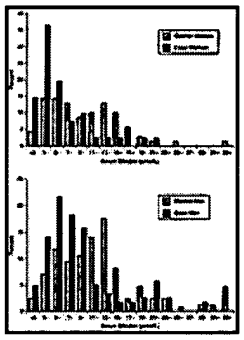

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Figure 1. Bar graphs show distribution of serum bilirubin concentrations in cases and control subjects.

To estimate the effect of different bilirubin levels on risk, we categorized cases and control subjects by quintile of serum bilirubin concentration (as determined among control subjects). Relative odds compared with the lowest quintile were calculated, as shown in Fig 2. There was a striking and progressive decrease in risk among both men and women as serum bilirubin levels increased. A 60% to 90% reduction in risk was apparent at higher quintiles.

The Mantel-Haenszel test for trend was highly significant in men (P=.00003) and women (P=.00002).

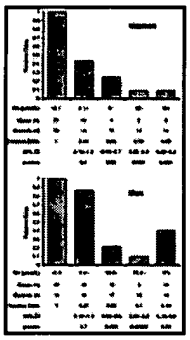

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Figure 2. Bar graphs show relative odds (compared with the lowest quintile) for increasing serum bilirubin (Bili) concentration. Quintile cut points were based on the distribution of serum bilirubin among control subjects. CI indicates confidence interval.

Multiple stepwise logistic regression was then performed with the following variables tested in the model, with presence of early CAD (case versus control) as the dependent variable: age, sex, body mass index, cigarette smoking, hypertension, diabetes, plasma total cholesterol, measured LDL cholesterol, triglycerides (natural log transformed), HDL cholesterol, and serum bilirubin concentration. Systolic and diastolic pressures were also tested in a separate model (data not shown), but the diagnosis of hypertension was associated with greater risk and precluded the entry of blood pressures into the model. Results of the multiple logistic regression are shown in Table 2. For bilirubin, a relative odds of 0.25 (95% confidence interval, 0.11-0.59; P=.0015) was associated with an increase of 17 μmol/L (1 mg/dL) in serum bilirubin concentration, a result remarkably consistent with the univariate calculation above. Results using log transformed bilirubin were virtually identical (not shown). Thus, the reduction in risk associated with increasing serum bilirubin appeared to be independent of other recognized coronary risk factors. The standardized regression coefficient for bilirubin (calculated by multiplying the nonstandardized coefficient by the SD of bilirubin) was similar to that for HDL cholesterol, suggesting that a 1 SD increase in either variable was associated with a similar degree of risk reduction.

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Table 2. Multiple Logistic Regression With Early Coronary Artery Disease (Case/Control) as the Dependent Variable

Because the patients with CAD were actually slightly younger than the control subjects at the time of their first diagnosis of CAD and many were being treated with lipid-lowering drugs, an analysis was performed without age as a variable and with the use of historical information. In this multiple logistic regression analysis, diabetes and hypertension were

considered present only if diagnosed by the time of CAD onset, and lifetime maximum reported total cholesterol (from questionnaires) was substituted for total measured cholesterol if it was higher than the measured total cholesterol at screening. Lifetime maximum total cholesterol entered first into this model (relative odds, 1.23; 95% confidence interval, 1.15 to 1.32; $P<.0001$), followed by HDL cholesterol and the other factors shown in Table 2. Risk estimates for these factors, including bilirubin, were essentially unchanged from values given in Table 2. In both models, sex was not a significant factor when HDL cholesterol and smoking entered into the equation. Logistic regressions performed separately for men and women showed virtually identical results for men (with bilirubin entering as a significant factor, $P=.0008$), whereas in the model for women, bilirubin was of marginal significance ($P=.051$) in all women but was highly significant when tested in nonsmoking women separately ($P<.01$). These results suggest that age differences in the cases and control subjects had little influence on the risk estimate for serum bilirubin. Among women, smoking appeared to modify the risks associated with serum bilirubin.

To further evaluate possible confounding, we examined univariate correlations between serum bilirubin and other variables. Although bilirubin did not correlate with age, body mass index, serum total cholesterol, LDL cholesterol, triglycerides, or blood pressure, there were significant correlations with HDL cholesterol ($r=-.18$, $P=.02$ in control subjects; $r=-.27$, $P=.0005$ in cases), albumin ($r=.28$, $P=.0004$ in control subjects; $r=.24$, $P=.003$ in cases), and creatinine ($r=.15$, $P=.06$ in control subjects; $r=.34$, $P=.0001$ in cases). In addition, cigarette smoking was associated with significantly lower serum bilirubin concentrations. Including these variables in a multiple logistic regression did not alter the strength of association between serum bilirubin and coronary risk (data not shown). Furthermore, in ANCOVA, the difference in mean serum bilirubin concentration comparing all cases with all control subjects remained highly significant after adjustment for sex, HDL cholesterol, albumin, creatinine, and cigarette smoking. Nevertheless, as shown in Fig 3, bilirubin levels in either former or current cigarette smokers (after adjustment for sex, albumin, creatinine, and HDL cholesterol) were not significantly different in cases and control subjects. Thus, most of the bilirubin effect was due to the striking differences seen between cases and control subjects who had never smoked.

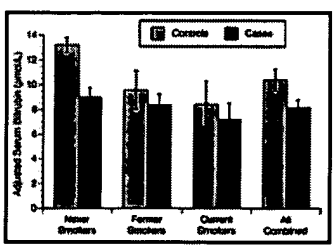

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Figure 3. Bar graph shows adjusted serum bilirubin concentration by cigarette smoking category.

In Fig 4, relative odds are shown separately for never- and ever-smokers. The protective effect of higher bilirubin levels remained clearly evident in the larger group of never-smokers. Although numbers are sparse for ever-smoking control subjects, there appeared to be some reversal of the protective effect of bilirubin among people with a positive smoking history.

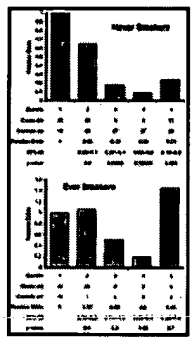

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Figure 4. Bar graphs show relative odds (compared with the lowest quintile) for increasing serum bilirubin concentration by cigarette smoking. Quintile cut points were based on the distribution of serum bilirubin among control subjects in men and women as in Fig 2. CI indicates confidence interval.

Discussion

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In this investigation we have confirmed and extended a previous observation that increases in serum bilirubin concentration within the normal range are associated with a significant and marked reduction in CAD risk. We observed a 60% to 90% reduction in risk when serum bilirubin was in the upper two control quintiles compared with the lowest quintile for both men and women. This risk reduction was independent of other known CAD risk factors and covariates. The apparent protective effects of bilirubin were of a similar magnitude as HDL cholesterol in this population. Much of this effect appeared to be restricted to nonsmokers, with reduced serum bilirubin found among smokers.

The single previously available epidemiological study of serum bilirubin in relation to CAD involved male Air Force pilots who had been referred for angiography because of positive or equivocal results in noninvasive testing. Those having angiographically defined coronary artery stenoses of 50% or greater had lower bilirubin levels than those who had normal coronary arteries (stenosis <10%). Furthermore, in multiple logistic regression after adjustment for other cardiovascular risk factors, a 50% decrease in total bilirubin was associated with a 47% increased risk of having more severe CAD (P=.02).[18] This predicted effect of serum bilirubin on CAD risk is somewhat less than our own. Differences in study design may in part account for the steeper risk gradient seen in our study. Our ascertainment of early familial CAD cases may select for those who are most vulnerable, and hence, those

with the fewest natural defenses against disease. If a higher serum bilirubin level is indeed a natural protective factor, then average levels would be expected to be more markedly decreased in a group with generally higher risk.

Several laboratory investigations have provided some biological plausibility for bilirubin acting as an antiatherogenic factor. Stocker et al[1] were the first to demonstrate antioxidant properties that may have physiological significance. Bilirubin and biliverdin at concentrations of 10 to 50 μmol/L were found to prevent formation of linoleic acid peroxides on exposure to a radical initiator in a homogeneous chloroform solution or in phospholipid liposomes. Bilirubin was more effective as an antioxidant at low oxygen tensions; at 2% oxygen, bilirubin suppressed oxidation more effectively than α-tocopherol. In a subsequent study, albumin-bound bilirubin was found to effectively inhibit peroxidation of albumin-bound fatty acids and inhibited oxidation of albumin itself. Albumin-bound bilirubin was converted to biliverdin on oxidation, quenching 2 mol of peroxyl radicals for each mole of bilirubin consumed.[2] Bilirubin and more especially albumin-bound bilirubin were found to be cytoprotective to rat hepatocytes, human erythrocytes, and human myocytes when these cells were exposed to oxyradicals.[4] [8] [9] Importantly, the ability of bilirubin at physiological concentration to effectively prevent oxidation of LDL lipids was recently demonstrated.[10] In another study, peroxidation of LDL lipids and vitamin E in fresh human plasma incubated with a constant source of peroxyl radicals occurred only after ubiquinol-10, vitamin C, and bilirubin were initially consumed. Furthermore, continued peroxidation of LDL lipid and vitamin E was strongly inhibited when exogenous bilirubin was added back into the oxidizing mixture. However, bilirubin no longer inhibited oxidation of LDL lipids once the LDL vitamin E was completely depleted.[11]

Our current findings and those of Schwertner et al[18] represent retrospective analyses of coronary cases and control subjects. In any case-control study it is not possible to be certain that the disease itself does not alter the risk factor in question. In two investigations of serum enzymes and bilirubin after acute myocardial infarction, there was a slight initial rise in serum bilirubin followed by a return to normal within 10 days.[33] [34] No long-term studies were found in a literature search. We cannot exclude the possibility that serum bilirubin declines after onset of CAD. Among a select group of neonates in whom enteral feeding had been withheld, babies with illnesses expected to cause increased oxidative stress had smaller increments of serum bilirubin than similarly treated infants without such diseases.[35] If CAD were similarly associated with a higher production of free radicals, increased consumption of bilirubin might occur as a secondary result of the CAD. Certainly, a number of well-conducted prospective investigations of CAD have included serum bilirubin among their baseline blood measurements. These data will be of great interest in analyses examining the important issue of time sequence and will provide confirmation of the retrospective analyses performed to date.

Cigarette smoking increases oxidative stress, as manifested by increased levels of a marker of lipid peroxidation in plasma of cigarette smokers.[36] Exposure of LDL to cigarette smoke

results in oxidation of lipids and greater uptake of modified LDL by macrophages.[37] [38] The lower serum bilirubin levels associated with cigarette smoking in our study may be due to oxidation of bilirubin by reactive species generated in cigarette smoke. Several other large population surveys have reported mildly reduced bilirubin levels in the serum of cigarette smokers.[39] [40] [41] These findings suggest that a possible additional mechanism whereby cigarette smoking increases the risk of CAD is by decreasing serum bilirubin. We cannot explain our observation that serum bilirubin was similarly reduced in former as well as current cigarette smokers. Perhaps an examination of prospective data could provide further insights regarding this question.

Interestingly, serum bilirubin levels were found to be higher in vegans than nonvegetarians.[42] Possibly the vegans' lower caloric intake may have resulted in higher bilirubin levels because fasting is well known to result in higher bilirubin levels.[43] Higher altitudes have also been associated with increased bilirubin levels apparently because of increased bilirubin production in association with the expected hematopoietic response.[44]

In conclusion, we have confirmed a strong inverse association between serum bilirubin and risk for early familial CAD. The reduction in benefit among current or former cigarette smokers may be consistent with an antioxidant role for bilirubin. These retrospective findings urgently need confirmation by prospective analyses.

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Footnotes

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